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Abstract: There is a lack of relevant prognostic and predictive factors in neurooncology besides mutation of isocitrate dehydrogenase 1, codeletion of 1p/19q and promoter hypermethylation of O (6)-methylguanine-DNA-methyltransferase. More importantly, there is limited translation of these factors into clinical practice. The cancer genome atlas data and also clinical correlative analyses suggest a pivotal role for the epidermal growth factor receptor /protein kinase B/mammalian target of rapamycin (mTOR) pathway in both biology and the clinical course of gliomas. However, attempts to stratify gliomas by activating alterations in this pathway have failed thus far. The tumors of 40 patients with WHO grade II gliomas without immediate postoperative genotoxic treatment and known progression and survival status at a median follow-up of 12.2 years were analyzed for expression of the mTOR complex 2 downstream target N-myc downstream regulated gene (NDRG)1 using immunohistochemistry. Baseline characteristics for NDRG1 absent/low versus moderate/high patients were similar. Time to reintervention was significantly longer in the NDRG1 group ($P = 0.026$). NDRG1 may become a novel biomarker to guide the decision which WHO^{II} glioma patients may be followed without postsurgical intervention and which patients should receive genotoxic treatment early on. Validation of this hypothesis will be possible with the observational arm of the RTOG 9802 and the pretreatment step of the EORTC 22033/26032 trials.

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NDRG1 prognosticates the natural course of disease in WHO grade II glioma

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Running Title:

NDRG1 is a biomarker in WHO grade II glioma

ABSTRACT

Background

There is a lack of relevant prognostic and predictive factors in neurooncology besides mutation of *isocitrate dehydrogenase (IDH)1*, codeletion of 1p/19q and promoter hypermethylation of *O⁶-methylguanine-DNA-methyltransferase (MGMT)*. More importantly, there is limited translation of these factors into clinical practice. The Cancer Genome Atlas (TCGA) data and also clinical correlative analyses suggest a pivotal role for the epidermal growth factor receptor (EGFR)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway in both biology and the clinical course of gliomas. However, attempts to stratify gliomas by activating alterations in this pathway have failed thus far.

Patients and methods

The tumors of 40 patients with WHO grade II gliomas without immediate postoperative genotoxic treatment and known progression and survival status at a median follow-up of 12.2 years were analyzed for expression of the mTOR complex (mTORC)2 downstream target N-myc downstream regulated gene (NDRG)1 using immunohistochemistry.

Results

Baseline characteristics for NDRG1 absent/low versus moderate/high patients were similar. Time to reintervention was significantly longer in the NDRG1 group ($P=0.026$).

Conclusions

NDRG1 may become a novel biomarker to guide the decision which WHO°II glioma patients may be followed without postsurgical intervention and which patients should receive genotoxic treatment early on. Validation of this hypothesis will be possible with the observational arm of the RTOG 9802 and the pretreatment step of the EORTC 22033/26032 trials.

Key Words:

Brain tumor, biomarker, glioma, NDRG1, prognosis

INTRODUCTION

Mutations of the *isocitrate dehydrogenase (IDH) gene 1* were first identified in human gliomas as a result of a large-scale genomic sequencing approach [1]. This has been confirmed across gliomas of World Health Organization (WHO) grades II-IV, including both astrocytic and oligodendroglial tumors [2-4]. However, most studies have failed to link *IDH1* mutations to better responsiveness to specific types of treatment, neither in glioblastoma [5], anaplastic glioma [2,6] nor low-grade glioma [7].

Although diagnoses of gliomas are based on histology, molecular abnormalities known to occur in these tumors have been evolving as supportive markers to assist diagnostics and patient management. Besides *IDH1* mutations, these markers in WHO grade II gliomas are *O*⁶-methylguanine-DNA methyltransferase (*MGMT*) promoter methylation status, deletions involving chromosomes 1p and 19q, and mutations in the *v-raf murine sarcoma viral oncogene homolog B1 (BRAF)* oncogene have moved into the focus of scientific interest and are already implemented in routine diagnostic workup. To evaluate the clinical relevance of these markers, it is crucial to distinguish the prognostic significance, defined as overall better outcome irrespective of management, from the predictive significance, defined as a better outcome given a specific treatment is administered [8].

Hypermethylation of the *MGMT* gene promoter for malignant gliomas and codeletion of 1p/19q for anaplastic oligodendroglial tumors are the only biomarkers linked to sensitivity to a specific treatment, that is, alkylating chemotherapy, specifically temozolomide (TMZ) for *MGMT* [9,10] and the combination of procarbazine, 1-(2-chlorethyl)-3-cyclohexyl-1-nitrosourea (CCNU) and vincristine (PCV) for 1p/19q codeletion [11,12]. Until recently, it was believed that the predictive value of *MGMT*

promoter methylation may be limited to glioblastoma since anaplastic gliomas showed a more favorable course of disease with a hypermethylated *MGMT* promoter in both alkylator- and also radiotherapy-treated patients [2, 11]. Interaction analyses between *IDH1* and *MGMT*, however, have revealed that *MGMT* promoter methylation is probably a predictive biomarker for response to chemotherapy in patients with *IDH1* wild-type, but not *IDH1* mutant gliomas, irrespective of WHO grade [13]. All currently known biomarkers unravel their prognostic properties only if genotoxic treatment is used and none of the biomarkers discussed so far, including *IDH* or *TP53 status*, *MGMT* promoter hypermethylation or 1p/19q codeletion, show a good relation to the natural course of disease [8,14]. In low-grade gliomas, guidance whether a patient with a newly diagnosed tumor needs early genotoxic treatment or not is pivotal, as the most commonly used treatment, radiotherapy of the involved part of the brain [15], is not associated with an overall survival (OS) benefit but possibly with long-term adverse sequelae especially in patients with a favorable prognosis [16].

The mTORC2-pathway effector, N-myc downstream regulated gene (NDRG)1, has been proposed as a prognostic factor in tumors including gliomas [17], although prognostic relevance in either direction has been described. Most recently, recurrent translocations of the oncogene plasmacytoma variant translocation (PVT)1, including PVT1-MYC and PVT1-NDRG1, have been described as potential targets in group 3 medulloblastoma [18].

Initial experiments assessing the role of NDRG1 in basic *in vitro* and *in vivo* proliferation paradigms surprisingly revealed growth-limiting properties of NDRG1 expression [19]. From that, we hypothesized that World Health Organization (WHO) grade II gliomas with a differential postoperative course of disease can be separated

on the basis of the expression of the mTORC2 downstream target, NDRG1, which then may serve as a biomarker for the decision to treat or to perform a watch-and-wait strategy.

Materials and methods

Patients and evaluations – German Glioma Network (GGN) cohort

The GGN is a prospective cohort study that enrolled newly diagnosed patients with various types of glioma and frozen tissue asservation from October 2004 to October 2010. From this cohort, we identified 40 patients with a diagnosis of a supratentorial WHO grade II astrocytoma or oligodendroglial tumor confirmed by history taking and central pathology review [20], as well as adequate follow-up at least until progression, who were followed by a watch-and-scan policy according to investigators' discretion supported by risk factor profiles [21]. Some of these patients were included in previous publications addressing the same clinical correlative question, but assessing different markers [8,14]. Clinical data were prospectively assembled as outlined before (<http://www.gliomnetzwerk.de>) [5]. All patients gave written informed consent. All activities of the GGN have been approved by the review boards of the participating institutions.

Molecular methods

1p/19q deletion status, *MGMT* promoter methylation as well as *IDH* and *TP53* mutational status have been determined as described previously [8,14].

Immunohistochemistry

Formalin-fixed paraffin-embedded tissue of 40 human grade II gliomas from the German Glioma Network (GGN) [8,14] was employed for immunohistochemical analysis for NDRG1 (goat anti-NDRG1 at 1:1000, Abcam, Cambridge, England, UK) on 3 µm sections. For quantitative analysis of the staining pattern by an independent neuropathologist blinded to the clinical data, the Histo-Score by Bruna *et al.* was

applied [22]. The score ranges from 0 to 300 and is calculated as the percentage of weakly stained cells plus the percentage of moderately stained cells multiplied by two plus the percentage of strongly stained cells multiplied by three. The entire tumor section was assessed except for areas not distinguishable from reactive tissue (Fig. 1). Ki67 immunostaining was done with an anti-Ki67 (MIB-1, mouse monoclonal antibody, Flex TRS low, 1:200 dilution, Dako, Glostrup, Denmark). For assessment of the proliferation index, anti-Ki67 immunostained tumors sections were scanned at low magnification to identify an area with high labelling density. In this area, 500 tumor cell nuclei were evaluated. The fraction of labelled nuclei per 500 tumor cell nuclei was determined by manual counting and is expressed as a percentage.

Statistical analysis

Survival data for NDRG1 low (0-10) or intermediate/high (>10-300) were plotted by the Kaplan-Meier method and analyzed by the log-rank test. Multivariate analysis of progression-free survival (PFS) used Cox proportional hazards models to adjust for the following clinical data and molecular markers: histological tumor type, resection status, age in years, *IDH1* status and *MGMT* promoter methylation status. Survival analyses were performed by B.H. using IBM SPSS Statistics Version 20.

RESULTS

Baseline clinical and molecular characteristics and NDRG1 expression

To assess whether the postoperative course of disease in WHO grade II glioma patients shows an association with NDRG1 expression, we took advantage of a series of patients who received no immediate radiotherapy or chemotherapy after the diagnosis had been established as part of a surgical intervention. The patients were assigned to two different groups: NDRG1 absent/low versus NDRG1 moderate/high as illustrated in Fig. 1. The two groups were well balanced with respect to age, post-operative KPS, histological subtype, Ki67 labelling, 1p/19q status, IDH status, TP53 status, but there was an overrepresentation of gross total resections and *MGMT* promoter hypermethylation in the NDRG1 absent/low group (Table 1).

High NDRG1 levels are associated with longer time to intervention, but not overall survival

After a median follow-up of 146 months, 40 patients have progressed (100%) and 26 patients (65%) have died. At progression, 24 patients were treated with radiotherapy, 10 patients with chemotherapy, and 6 patients with combined modality treatment.

In the univariate analysis oligodendroglial histology and moderate/high NDRG1 expression were associated with a strong risk reduction for progression (Table 2). Moderate/high NDRG1 expression was associated with superior PFS [NDRG1 low: median PFS 30 months (95% confidence interval 12-48 months)] *versus* [NDRG1 medium/high: median PFS 54 months (95% confidence interval 24-84 months)] (Fig. 2a). Of note, the effect of NDRG1 was present also in analyses separated for astrocytic and oligodendroglial histologies without differential impact. On multivariate analysis, oligodendroglial histology (HR 0.3 95% CI (0.1-0.6), $P=0.001$) and

moderate/high NDRG1 expression (HR 0.4, 95% CI (0.2-0.8, $P=0.01$) persisted as strong prognostic factors (Table 2).

For OS, however, i.e. after one or more genotoxic treatments, which included sequential radiotherapy and alkylating chemotherapy in either order in all patients, only age was prognostic (<40 years versus ≥ 40 years, $P<0.05$) and the two NDRG1 groups did not differ anymore (Fig. 2b). Due to the lack of archival data to confirm our findings, we just took the opportunity to check for the potential negative impact on OS in patients that had undergone radiochemotherapy. Analysis of the REpository for Molecular BRAin Neoplasia DaTa (REMBRANDT) database revealed that the OS of glioblastoma patients with intratumoral upregulation of *NDRG1* was reduced compared to patients with intermediate or downregulated expression of this gene (Fig. 2c).

These data indicate that moderate/high NDRG1 expression is an independent prognostic factor for favorable outcome in patients with WHO grade II gliomas not exposed to postsurgical treatment.

DISCUSSION

Despite relevant progress in the field of molecular classification of malignant gliomas, all attempts to define a parameter, which predicts the natural course of disease in low-grade gliomas, have failed thus far. Patients with these tumors are currently treated or followed based on the set of prognostic parameters developed from the EORTC 22844 trial [21], but still a lot of uncertainty for individual treatment decisions remains. From this set of parameters, oligodendroglial histology has been confirmed here as prognostic for PFS without postsurgical intervention (Table 2).

Analyses of the prognostic potential of 1p/19q codeletion, *IDH1* mutation and *MGMT* promoter hypermethylation revealed a prognostic or predictive potential of these markers only when radio- or chemotherapy were used [5,8,14]. In addition, there are molecular alterations like duplications or mutations of the *v-raf murine sarcoma viral oncogene homolog B1 (BRAF)* that not only define a specific glioma entity, but may also be linked to a specific therapy [23]. There is an unmet need for molecular markers with prognostic impact in the immediate postsurgical situation [24] that facilitate daily decision-making in the clinic.

Despite its name, *NDRG1* is neither regulated by nor correlated to N-Myc in glioma [19]. *NDRG1* has been described as a hypoxia-inducible protein in glioblastoma, whose expression is associated with increasing malignancy in gliomas, peaking out in relapsed glioblastomas following radiochemotherapy [19]. Our survival analysis on postsurgically untreated WHO °II gliomas demonstrate an *NDRG1*-associated growth delay and thus an improved PFS in a multivariate analysis (Fig. 2a), which was not visible any more at OS when genotoxic treatments had been applied (Fig. 2b). Due to a lack of a confirmatory data set, we only confirmed the negative prognostic impact of *NDRG1* mRNA expression when treatments are applied (Fig.

2c). These data allow a separation of disease- and treatment-modifying effects and support a tumor-suppressing function of NDRG1, which is limiting tumorigenicity in the natural course of the disease. Further, the other biomarkers discussed in glioma, 1p/19q, *IDH1*, *MGMT*, Ki67 labelling [8,14], hot spot mutations in the promoter region of telomerase reverse transcriptase (TERT) [25] and mutation/loss of alpha-thalassemia/mental retardation syndrome X-linked (ATRX) expression [26] show a good relation to the natural course of disease, but unravel their prognostic properties only if cytotoxic treatment is applied. This prevents their use to categorize tumors and also to determine more favorable subgroups of tumors, in which a wait-and-scan strategy after surgery might be better than immediate cytotoxic treatment. The size of our patient cohort prevents further subgroup analyses. In this cohort, no interaction between tumor type, 1p/19q codeletion, *IDH1* mutation or *MGMT* promoter methylation and NDRG1 expression was found. Of note, although no significant differences are found between the two NDRG1 expression groups, positive risk factors, specifically gross total resection, are numerically higher represented in the NDRG1 no/low group, and therefore bias against our finding (Table 1).

Outside the brain tumor field, the relevance of NDRG1 depends on the tumor entity. NDRG1 suppresses the proliferation of breast cancer cells *in vitro* [27] and the tumor growth of pancreatic and bladder cancer *in vivo* [27,28], whereas survival and proliferation of hepatocellular carcinoma cells are promoted [29]. In line with these preclinical findings NDRG1 expression is associated with a favorable prognosis in patients with breast, colon or pancreatic cancer [28,30,31], but associated with more aggressive disease in cervical or liver cancer [32,33]. In these studies, effects on tumor growth and therapy-modifying properties of NDRG1 are not separable.

In summary, NDRG1 may serve as the first prognostic biomarker for the natural course of disease in WHO grade II gliomas that awaits validation in the analyses of the observational arm of RTOG 9802 and the registration step of the EORTC 22033/26032 WHO grade II glioma trials.

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CONFLICT OF INTEREST

None.

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FIGURE LEGENDS

Figure 1. Histoscore for NDRG1

(a) NDRG1 immunohistochemistry in a case of moderate staining (histo score group >10, left) and a case with absence of NDRG1 expression (histo score group <10). Note staining of the tumor cell processes (arrows) and the decreasing staining of tumor matrix apart from strongly positive areas (arrowheads). (b) Representative cases illustrating the application of an adapted histo score²² for evaluation of NDRG1 staining on paraffin-embedded glioma sections. The score ranges from 0 to 300 and is calculated as the percentage of weakly stained cells plus the percentage of moderately stained cells multiplied by two plus the percentage of strongly stained cells multiplied by three (positive cells are stained red and an isotype control (c) is provided). The entire tumor section was assessed except for areas not distinguishable from reactive tissue.

Figure 2. NDRG1 expression prognosticates superior PFS in postsurgically untreated WHO grade II gliomas

(a) PFS and (b) OS data of 40 patients with a WHO grade II glioma without postsurgical treatment until further progression are plotted in the group no/low or moderate/high expression of NDRG1 in the tumor tissue. Scoring and grouping was done by an independent neuropathologist (FS) blinded to PFS or OS data. (c) NDRG1 expression relative to patient survival in glioblastoma (REMBRANDT).

Figure 1

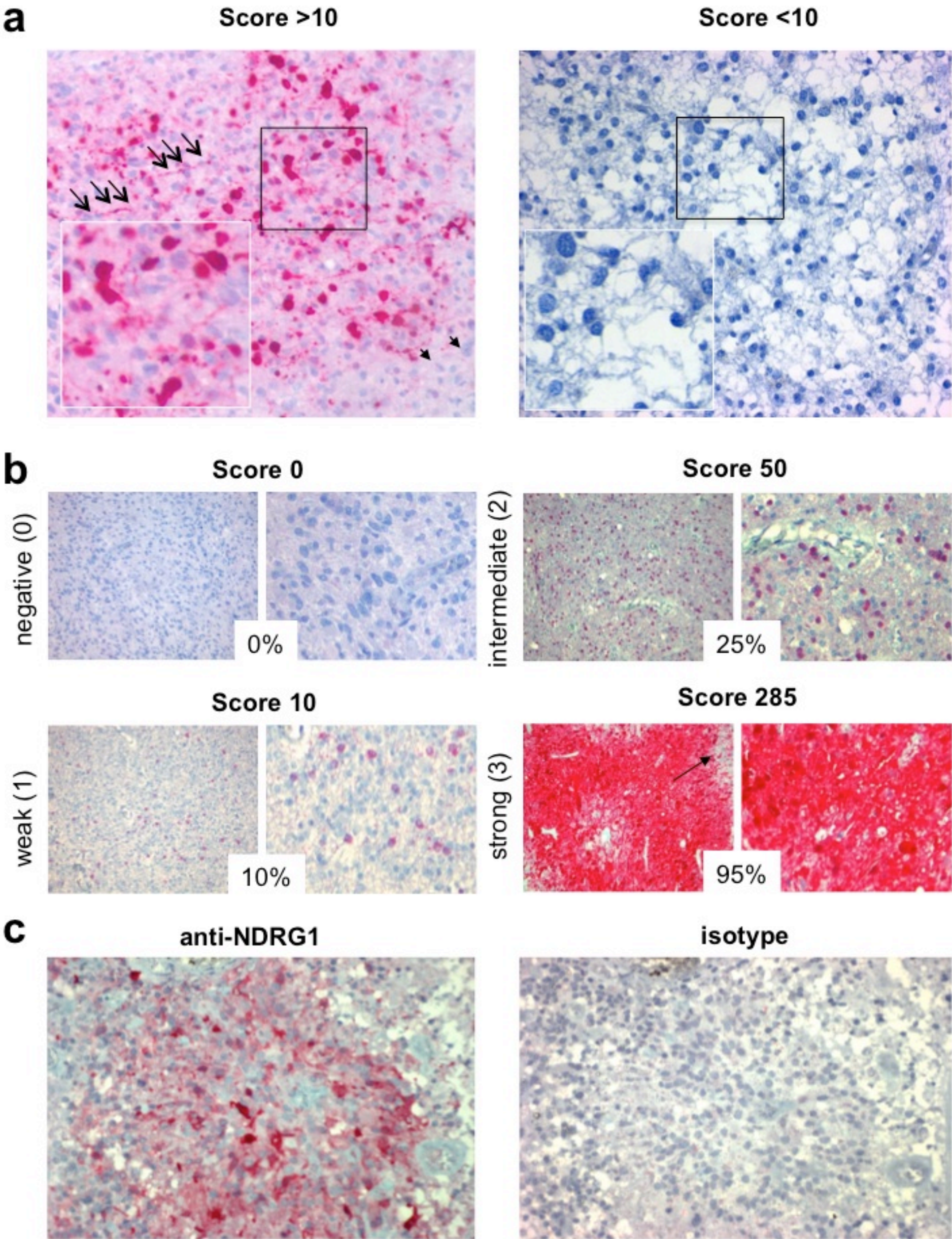


Figure 2

